AD NUMBER

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OCRD D/A ltr, 14 Jul 1972

Biochemical Toxicology of CA, CS, and CN

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I. INTRODUCTION.

The riot control agents are reactive chemicals capable of binding biological anions and initiating pain, bronchoconstriction, other physiologic responses and sometimes death. This study attempts to relate the reactivity of these agents to their systemic toxicity and further define the mechanism of their mode of action and elimination.

II. MATERIALS AND METHODS.

A. Systemic Toxic Effects

The toxicity of CA, CS, and CN were determined in Sprague-Dawley rats, ICR mice and N.Z. white rabbits by the intravenous, intraperitoneal and oral routes. The LD50 and corresponding 95% confidence interval was derived by the method of Bliss.[1]

For toxicity determination, the agent was dissolved in PEG 200 at a concentration of 20 mg/ml. Aliquots were measured for injection in graduated syringes as a function of individual body weight. CA was a repurified sample obtained from Mr. Stanley Kramer (WDEL-CP Labs). Melting point approximately 20°C. The crystals were only mildly discolored and its solutions were clear when initially prepared. Only colorless solutions were used since discoloration was noted on standing. CS was obtained from Mr. T. Ballard (Aerosol Branch). CN was Eastman chloracetophenone Lot 832.

B. Nucleophilic Reactivity

In vitro nucleophilic reactions were measured in a recording spectrophotometer (Gilford 2000) at 37°C. The alkylating agent was added to solutions of nucleophilic reagents (20:1 in molar excess) made up in 0.1 M pH 7.2 phosphate buffer. The concentration of the alkylating agents were in the µg/ml range due to their relatively low water solubility. The wavelengths employed for CA, CS, and CN were 254, 300 and 249 mµ respectively. The reaction constants derived from pseudo-first order kinetics were obtained directly from the rate of change of the optical density with respect to the initial reading.

Lactic dehydrogenase (LDH) and nucleotide adenine dinucleotide (NAD) activities were determined as described in Sigma Bulletin 340 UV. The enzyme or NAD was incubated with CS or CN at 37° for periods up to 16-18 hrs. Glutathione was added to the incubation mixture in order to stop the reaction and consume any excess alkylating agent.

In vivo reactions were demonstrated by parallel experiments to the in vitro studies. This portion of work employed either BAL or thiosulfate injected parenterally in rats at predetermined safe dose levels.

C. Detoxication

Thiocyanate and cyanide ions were analyzed by the method of Bruce.[2] Sulfhydryl concentrations were measured by the methods of Harrap[3] and Flesch et al.[4] Urinary SO_4 was measured by a nephalometric method using a Aminco Bowman Spectrofluorometer with excitation and emission at 450 mµ.[5]

D. Physiologic Response from Aerosols

Mongrel dogs of either sex were anesthetized with pentobarbital Na, 5 mg/kg administered intravenously. Some dogs were intubated with a cuffed endotracheal tube. The CS aerosol from a M7A2 grenade was delivered from a mixing chamber to the lung by means of the endotracheal tube by-passing the upper respiratory tract. This method allowed continuous monitoring of response but limited the deliverable concentration.

Other dogs were placed inside the chamber for total body exposure. The concentrations were higher in this case but physiological measurements were not made until after removal from the chamber.

Blood pressure, pulmonary ventilation, and venous pressure recordings were made on an E-M Physiograph.

Blood pH, pCO_2, and pO_2 were determined on a Instrumentation Laboratory blood pH meter and polaragraph. Dogs were observed until no further changes were recorded. Blood clotting was determined in all siliconized glassware at room temperature.

E. Other Metabolic Disturbances

In order to determine micro-humeral response, rat lung was profused with Tyrode's solution through the trachea. The rate of flow and pressure through the isolated lung was measured. Histamine was determined by the method of Shore.[13]

Bradykinin-like materials were measured by the method of Horton for guinea pig ileum.[12]

Pentobarbital sleeping time in mice was measured by the duration of loss of righting reflex. At the dose used (25 mg/kg, intraperitoneally) control animals never lost their righting reflex.

III. RESULTS.

A. Nucleophilic Reactivity

The alkylating agents react spontaneously in vitro with a variety of nucleophilic anions (Table 1). In addition LDH, a sulfhydryl dependent enzyme, has been shown to be depressed by CS and CN (Table 2). Even the co-factor NAD is affected after incubation with the agents.

Studies in rats indicate that sulfhydryl anions administered to rats before giving CA, CS, or CN protect the animals and prolong survival (Table 3).

Usually, if the nucleophilic compound is given after the alkylating agent no protection is afforded the animal. However, rats prostrate after a lethal dose (125% LD50) of CS may survive if given thiosulfate intravenously (Table 4).

This same reversibility of the activity of the CS-inhibition of LDH and NAD may be demonstrated in vitro with a physiological nucleophile such as glutathione. However, with CN inactivation the enzyme and co-factor are refractory to glutathione reactivation.

Further evidence for an in vivo attack on nucleophilic sites is seen in dogs given CA, CS, and CN intravenously. With each agent the sulfhydryl level in the plasma are depressed and returns slowly to a control value (Fig #1).

The importance of the body sulfur pool with respect to the toxicity of these agents may be demonstrated by its depletion and observing the resulting sensitivity. The urinary excretion of SO_4 was 7 to 20 µgm of SO_4 per day in four rats during a three day control period. After two doses of CS 20 mg/kg orally five days apart, the excretion of SO_4 in all four rats dropped to zero. Five days after the second dose of CS a single iv injection of 15 mg/kg (1/2 LD50) caused immediate death in all animals.

Products of the in vitro reaction of CS with various nucleophilic anions have been isolated. It has been shown that CS will undergo a series of reversible reactions with mercaptoethanol-diethylamine.[6] A product of this reaction, CS:(DEAEM) given to rats in molar amounts equivalent to a toxic dose of CS produced no toxic effects. The product of the reaction of CS and cysteine was also without toxicity.

B. Metabolic Products

The CS anion reaction product is further degradated in the body to thiocyanate and organic residues. Although 70% of the nitriles of CA appear in the urine as thiocyanate, only 15 to 30 percent of the nitriles from CS are recovered as thiocyanate.

When the CS:(DEAEM) conjugate was given intravenously to a rat, the 30 min blood level of thiocyanate was 5.0 μg/ml which is approx 30% of the nitriles on the parent compound.

However, if the CS-cysteine product is given to rats orally or intravenously, no thiocyanate is recoverable.

Thiocyanate also appears in the blood of dogs exposed to CS aerosol from grenades (Fig 2).

C. Physiologic Effects

The physiology of CA, CS, and CN are qualitatively very similar when given by any one route. However, different methods of administration elicit different responses and toxicities.

Intravenous CA, CS, and CN to rats causes hyperactivity, convulsive dashing about and finally collapse. Animals may then either completely recover or die (Table 5).

When the agents are given intraperitoneally only mild writhing occurs apparently from pain. Given orally, nothing is seen except gradual weakening and death overnight.

With intravenous CS administration in dogs, death results from cardiac arythemia. The animal is able to consume extremely large amounts of CS by this route before death, which occurred with doses of 30 mg/kg over a 5 hr period. During this time the arterial pO_2, and pH remained constant but the pCO_2 decreased from 36.2 mm Hg to 20.5 mm Hg. No change in hematocrit was observed.

In the exposure of dogs to CS aerosol one observes an initial response of apnea followed by slow prolonged inspiratory and expiratory bronchoconstriction with episodic hyperventilation. The body temperature falls (Fig 3). An increase in blood pCO_2 and hematocrit together with a depression of pO_2, pH and clotting time was observed. (No depression of plasma sulfhydryl has yet been observed from aerosol exposure). The blood pressure was mildly elevated but no signs of abnormality of the venous pressure or EKG occurred.

Usually the animal survives this insult but some expired 48-72 hrs later from atelectasis and pulmonary edema.

The lachrymator agents increase susceptibility of mice to barbiturate and cyanide. Mice given CS either intraperitoneally or by aerosol have markedly prolonged sleeping times following a standard dose of pentobarbital (Table 6).

Rats showed an increased susceptibility to cyanide poisoning following CN given parenterally (Table 7). These altered reactions do not appear to be due to altered liver blood flow since BSP disappearance was not depressed in mice under similar conditions.

There appears to be an histamine effect in the response of animals to CS. Mice given histamine intravenously have an augmented response to intravenous CS (Table 7).

In addition, the bronchoconstriction in dogs to CS aerosol is relieved by a dimenhydramine (Benedryl).

IV. DISCUSSION.

A. Nucleophilic Reactions

All the lachrymators studied are alkylating agents which react in vitro with biological anions (Table 1). They also appear to react in vivo as measured by suppression of plasma sulfhydryl levels after administration of CS or CN intravenously (Fig 1) and (Table 2). This evidence, together with the fact that exogenous nucleophilic compounds protect animals from the toxicity of CA, CS, and CN support the thesis that the mechanism of toxicity of these substances is an attack on essential nucleophilic enzymes in the body.

There is no data on the precise biochemical lesion caused by these compounds. Peters[16] has suggested that pyruvate decarboxylase and more precisely dihydrolipoic acid may be the prime target but no conclusive evidence is available. Sacktor[14] has shown that glyceraldehyde PO_4 dehydrogenase is inhibited by CS. Trypsin was inhibited 40% by 2.5×10^{-2} M CS in this laboratory. It is not possible then to attribute toxicity to any single enzyme system at the present time.

The relatively low toxicity of CS may be explained by the rapidity of the reaction of CS with water and other non-essential nucleophilic sites in blood and tissues. This has been described by Ross[7] as the "mopped up" effect. This might explain the 8-fold decrease in toxicity of oral CA and CS compared to only a 3-fold decrease with the slow reacting CN.

A second explanation of the low toxicity of CS is its unique quality of reversibility.[6] It has been demonstrated that the inhibition of CS upon LDH in vitro is reversed by the nucleophilic compound glutathione (Table 2). CN inhibition of this enzyme on the other hand was not reversed by glutathione. The in vivo correllary of the observations was the ability of intravenous thiosulfate to reverse toxicity after rats were symptomatic from a lethal dose of CS. Rats given a lethal dose of CN, however, received no benefit from thiosulfate treatment (Table 4).

B. Detoxication

After the initial nucleophilic reaction, the nitrile groups on the CS are cleaved off in the body. The free cyanides are then converted to the non-toxic thiocyanate. Only about 30% of the CS given to rats orally, intraperitoneally or intravenously appear in the urine as thiocyanate. We are unable to augment the yield of free cyanide from the CS by pretreatment of the animals with thiosulfate or by incubation of the CS with glutathione prior to injection.

In dogs the plasma levels of thiocyanate were followed after CS by the intravenous and inhalation routes (Fig 2). The thiocyanate levels reach an apogee in about 24 hrs and then slowly falls. Thiocyanate has a half life in the body of about 24 hrs and is excreted exclusively by the kidneys. The volume of distribution is about 300 ml/kg of body weight. Thus it is detectable in both plasma and urine for an extended time after exposure. This may make thiocyanate levels a convenient method for qualitative and quantitative estimation of CS exposure. Although the peak plasma concentrations of 6.64 mg/ml of thiocyanate after 8 mg/kg intravenously of CS is in fair agreement with a 30% recovery (6.64 mg/ml x 0.3 ℓ/kg x 100/30 8 mg/kg), there is no evidence that the yield of thiocyanate from CS aerosol is in the same order of magnitude as other routes.

C. Pathophysiology

Deaths from CS aerosol is caused by lung pathology. Pulmonary edema, atelectasis and hemorrhage together with secondary bacterial pneumonia were found 48 to 96 hrs post exposure.

It has not been possible to kill anesthetized dogs exposed for 25 mins to CS aerosolized from a grenade with concentrations of 2000 mg/cu m (Ct 50,000).

Attempts to study physiological responses in dogs at the time of death failed because of our inability to sustain a high enough concentration even with multiple grenade detonations.

With CN and DM, however, lethal concentrations were obtained while the animals were under study. Consequently we are compelled to draw relationships of the highest obtainable (but immediately non-lethal doses of CS aerosol to lower (but immediately lethal) doses of CN and DM.

Apnea is the characteristic response of an animal exposed to an irritant aerosol. Acid, ammonia, ozone, NO_2, SO_3, and "tear gas" alkylating agents all cause immediate suppression of respiration. Although the nasal mucosa is most sensitive to the irritants, the lower respiratory tract responds in a similar manner at least for CS. Respirations begin again 30 to 60 seconds post initial apnea despite the continued presence of the irritant. Although the respiratory rate may vary it is usually increased over control, but total ventilation is depressed with all irritants. CS aerosol has a peculiar property of causing episodic hyperventilation in dogs occurring every 15 min and lasting about 2 min. Episodic ventilation has not been seen with CN or DM aerosol or intravenous CS in any dose range. There is no obvious relationship of blood pH, pCO_2, or pO_2 to the hyperventilation but it may be stopped by intravenous dimenhydramine.

Low concentration levels of CS may cause enough hyperventilation to increase the arterial pO_2 above control (120 mm Hg over control of 90 mm Hg). However high exposure levels cause a depression of pO_2 (40 mm Hg) even in survivors (Fig 3). With DM and CN there is a consistant drop in the pO_2 of moribund animals; but survivors maintain a normal pO_2.

With depression of the pO_2 there is an elevation of the pCO_2 and blood acidity. This, however, is highly variable and two dogs exposed simultaneously in the same chamber may have markedly different pCO_2's. It is surprising that animals are able to survive after maintaining a blood pH of below 7.0 for 10 hrs.

CN and DM cause an increase in venous pressure. It is not established if this is an indication of congestive heart failure or pulmonary arterial constriction. The force of contraction in isolated dog heart preparation is decreased by CN and DM with increased back pressure. CS does not cause an increased venous pressure. Cardiac effects of CS are seen only with continuous intravenous infusion of lethal dose of CS. In dog and monkey there are EKG changes suggestive of hyperkalemia. That is to say an increase PR interval and disappearance of the P wave, decreased R wave and increase in S, peaked T wave and widened QRS pattern in Lead I. This was followed by asystoli and death. Plasma electrolytes at this time did not show a high potassium concentration but a consistent pattern of lowered Na ion concentration of approximately 20 meq/l in 20-30 min and a normal unchanged potassium. No change in electrolytes has been seen with CS aerosol.

It is felt then that CN and DM administered as aerosol may very well cause acute congestive heart failure but CS has not been shown to cause any heart disturbance.

Although the cardiopulmonary axis is the major site of the lachrymator toxicity other metabolic disturbances are seen.

The hematocrit consistantly increases with CS aerosol exposure, an increase of 20-50% over control is usually seen. This occurs despite repeated blood sampling (approx 10% of estimated blood volume) or splenectomy. It requires 24 hrs for the elevation to return to normal.

Dogs exposed to CS aerosol show a drop in body temperature. This is not seen when the agent is administered intravenously. Body temperatures as low as 76°F about 1 hr post exposure were recorded. These generally equilibrate to 85°F during the 10-hr observation period (Fig 3). The mechanism and significance of the drop in body temperature is unknown. It is possibly an attempt to decrease oxygen requirement. In any case the animals survive. Hypothyroid rats are known to be resistant to ozone toxicity and rats given thryroxin are more sensitive.[17]

The metabolism of barbiturates is dependent upon an intact hepatic glycogen and ample supply of NADPH. Since the riot control agents might affect these substances, animals exposed to CS were tested for their sensitivity to barbiturates.

There is an increase sleeping time of mice given pentobarbital after CS (Table 6).

This drug sensitivity was also noted for sodium cyanide in rats after given CN.

Since the routes of metabolism of cyanide and pentobarbital are thought to be entirely different and independent, it is hazardous at this time to suggest a common mechanism. BSP clearance in mice given CS was no different than control so a large alteration in hepatic blood flow is not likely to be the explanation. Therefore the observation that our pentobarbital anesthetized dogs required no supplementary anesthesia through 10 hrs of observation and were either asleep or dazed 24 hrs after exposure tends to support this conclusion.

It has been reported that the toxicity of certain alkylating agents (nitrogen mustard) may be increased by treating mice with histamine. In addition, the toxicity was decreased by antihistamine. Since the hyperventilation caused by CS aerosol was relieved by Benedryl, it is possible that this neurohumor may take part in the body's reaction to riot control agents.

Table 7 indicates that pretreatment of mice with histamine increases the toxicity of CS and CN.

CS is capable of causing the release of histamine from minced lung.[12] We have been unable to obtain histamine from either Tyrode perfused whole lung or dialysate of rat peritoneum with CS in saline solution (10 μg/ml). However, we have been able to obtain a "bradykinin" like substance from both preparations. The rapid clotting of blood obtained from animals exposed to CS and DM aerosol correlates with factor XII, the initiator of in vitro clotting. The factor XII of blood clotting is a "precursor" of bradykinin release.

The bronchoconstriction mediator of riot control agents may well be bradykinin-like rather than histamine.

V. CONCLUSIONS.

1. The lachrymator agents CA, CS and CN react in vitro and in vivo with biological anions. There is a definite relationship between the in vitro reactions of these agents and their toxicologic effects.

2. In the body the CS-anion complex is further degraded to yield thiocyanate and organic residues.

3. CS releases smooth muscle active substances from tissue not unlike bradykinin.

4. CS aerosol produces a somewhat unique syndrome in dogs characterized by apnea followed by episodic hyperventilation, erythrocytosis, hypothermia, anoxia and acidosis. Recovery by these dogs even from high doses (Ct's) is the rule.

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Table 1
First Order Reaction Constants of
Lachrymators with Excess Nucleophilic Agents

	Phosphate buffer pH 7.2	Glutathione	BAL	S_2O_3	Plasma protein	Dihydro-lipoic acid	Mercaptoethanol-diethylamine	Cleland's reagent
CA	.0012	1.16		.094				2.15
CS	.083	.95	0.46	.072	.57	10.4	1.57	0.76
CN	.00067	.17	0.46	.020		4.4	0.41	0.30

Table 2
Reaction of Riot Control Agents with LDH and NAD

	OD/min x 10^{+2}			
	no GSH	GSH added*		
LDH	0.875	0.86	LDH:	85 units/ml
LDH + CS	0.000	0.37	CS:	1.0×10^{3} M
LDH + CN	0.000	0.000	CN:	1.25×10^{-3} M
	no GSH	GSH added**		
NAD	1.64	1.50	NAD:	1.63×10^{-3} M
NAD + CS	0.000	1.00	CS:	1.0×10^{-3} M
NAD + CN	1.34	1.10	CN:	1.1×10^{-3} M

* Incubation time with GSH (1.3×10^{-2} M) 30 min.

** Incubation time with GSH (1.1×10^{-2} M) 10 min.

Table 3
The Effect of iv BAL on the Speed of Action of iv CA, CS, and CN in Rats

Agent* 40 mg/kg	BAL* 20 mg/kg	Time to Death min	Fractional Survivors
CA	no	30 sec	0/6
	yes	3-6 min	4/6
CS	no	75 sec	0/6
	yes	1-28 min	1/6
CN	no	2-12 min	1/6
	yes	0.N.	1/6

* Injection solvent - PEG 200; BAL administered 0.5 min before agent.

Table 4
Effect of Sodium Thiosulfate After CS Poisoning

Group	No. of animals	IP DOSE CS mg/kg	IP DOSE Sodium Thiosulfate mg/kg	Mortality Fraction	Time to Death
I	6	80	20	5/6	(3)< 24 hrs (2)> 48 hrs
II	6		40	5/6	(1)< 24 hrs (4)> 48 hrs
III	8		60	2/8	(1)< 24 hrs (1)> 48 hrs
IV	7		80	2/7	(2) 2 hrs
control	8	80		7/8	(2)9.8;(1)1 (3)1.7;(1)1.9
control	8		80	0/8	-

* Sodium thiosulfate was given when animals showed severe signs of CS poisoning.

** Figure in parenthesis denotes the number of animals dying at that time.

Table 5
Toxicity of CA, CS, and CN in Rats, Mice and Rabbits

	CA		CS		CN	
	No. of animals	LD50 mg/kg	No. of animals	LD50 mg/kg	No. of animals	LD50 mg/kg
Rat						
oral	42	411(335-504)	36	717(648-792)	42	127(120-135)
ip	42	52(45-61)	36	66(55-80)	42	56(49-65)
iv	36	34(32-36)	24	35(32-38)	24	35(33-37)
Mouse						
oral	50	201(176-229)	50	282(196-407)	40	139(126-155)
ip	60	43(39-49)	50	25(23-28)	40	60(55-65)
Rabbit						
iv	30	16(13-19)				

Table 6
Pentobarbital Sleeping Time in Mice with CS*

Time after dosing	CS-ip (10 mg/kg)	CS-aerosol (Ct 57,000)
min	minutes slept	
15	36 ± 5.7*	-
30	40 ± 5.7	43 ± 4.5
45		66 ± 13.7
60	49 ± 17.9	
75		49 ± 13.4
90	75 ± 30.7	39 ± 8.6
120	37 ± 6.4	41 ± 17.9
150	32 ± 3.2	-
24 hrs	-	-

* Standard deviation of mean based on 10 animals

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Figure 1

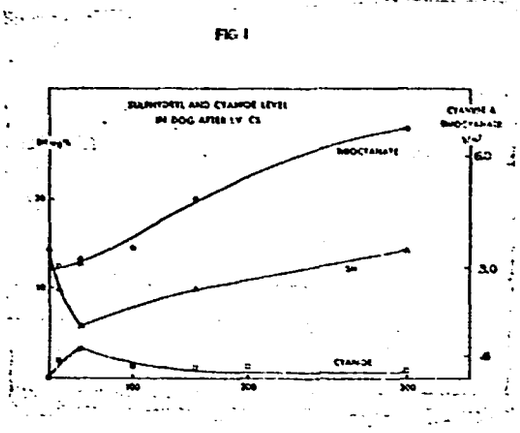

Figure 2

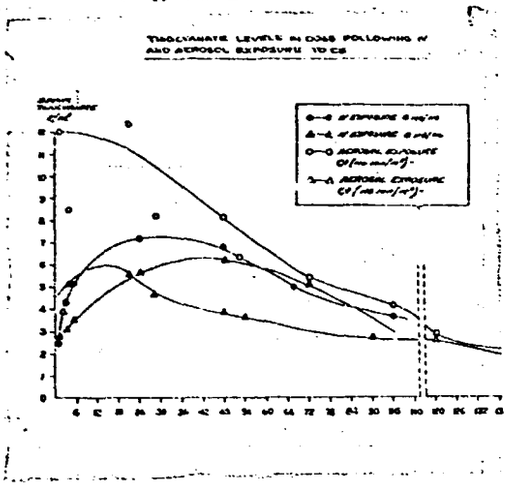

Figure 3

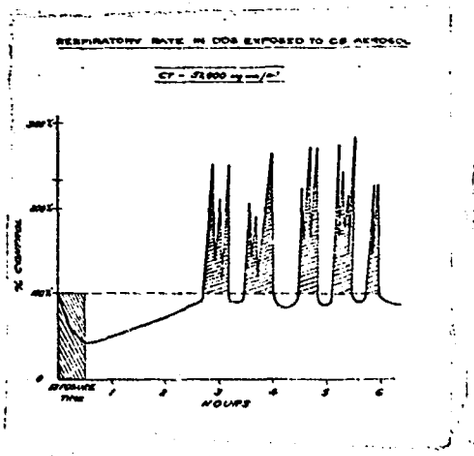

Figure 4